Article

Whole exome/genome sequencing joint analysis in a family with oligogenic familial hypercholesterolemia

**Abstract:** Autosomal Dominant Hypercholesterolemia (ADH) is a genetic disorder caused by pathogenic variants of the *LDLR*, *APOB*, *PCSK9,* and *APOE* genes. We sought to identify new candidate genes responsible for the ADH phenotype in patients without pathogenic variants in the known ADH-causing genes by focusing on a French family with affected and non-affected members who presented a high ADH polygenic risk score (wPRS). Linkage analysis and sequencing of whole exomes and genomes resulted in the identification of variants p.(Pro398Ala) in *CYP7A1*, p.(Val1382Phe) in *LRP6* and p.(Ser202His) in *LDLRAP1*. Six other variants were identified in six of 160 unrelated ADH probands: p.(Ala13Val) and p.(Aps347Asn) in *CYP7A1*; p.(Tyr972Cys), p.(Thr1479Ile) and p.(Ser1612Phe) in *LRP6;* and p.(Ser202LeufsTer19) in *LDLRAP1*. All six probands presented a moderate ADH wPRS. Serum analyses of carriers of the p.(Pro398Ala) variant in *CYP7A1* showed no differences in the synthesis of bile acids compared to the serums of non-carriers. Functional studies of the four *LRP6* mutants in HEK293T cells resulted in contradictory results. Within the family, none of the carriers heterozygous for only the *LDLRAP1* p.(Ser202His) variant presented ADH. Altogether, each variant individually does not seem to contribute sufficiently to the elevation of LDL-C, and it is the oligogenic combination of two or three variants that is necessary to reveal the ADH phenotype.

**Keywords:** Autosomal Dominant Hypercholesterolemia; Linkage analysis; Next generation sequencing; LDL uptake; *CYP7A1*; *LRP6*; *LDLRAP1*; Protein structural models; Polygenic risk score; Oligogenic hypercholesterolemia.

1. Introduction

Autosomal Dominant Hypercholesterolemia (ADH) is a genetic disorder affecting lipoprotein metabolism that is characterized by high plasma levels of low-density lipoprotein (LDL) due to its reduced catabolism [1]. Lifelong exposure of arteries to elevated levels of cholesterol promotes early atherosclerotic plaque development and premature cardiovascular disease (CVD) increasing the risk of heart attack, stroke and peripheral vascular disease [2]. Besides CVD, individuals with ADH may have extravascular deposits such as tendinous xanthomas, xanthelasma or corneal arcus [3].

ADH is one of the most frequent genetic diseases with a prevalence of 1 in 313 in the general population [4], and more frequent in populations with founder effects (i.e. French Canadians, Afrikaners, Lebanese) [5]. ADH is caused by mutations in the low-density lipoprotein receptor (*LDLR)* gene at 19p13.3 (OMIM #143890, #606945) [6,7], the apolipoprotein B (*APOB)* gene at 2p23–p24 (OMIM #107730, #144010) [8], the proprotein convertase subtilisin/kexin type 9 (*PCSK9 )*gene at 1p32.3 (OMIM # 607786) [9] and the apolipoprotein E (*APOE)* gene at 19q13.32 (OMIM #107741) [10]. The low-density lipoprotein receptor adaptor protein 1 (*LDLRAP1)* gene at 1p36.11 (OMIM # 605747) is the only gene responsible for the recessive form of hypercholesterolemia identified to date [11].

Mutations in the *LDLR* gene are the most frequent cause of ADH (80 – 85% of the cases), and more than 3000 variants have been reported. The LDLRL removes LDL particles from plasma, and alterations in the receptor are commonly associated with high LDL-C levels [3]. Mutations in *APOB* are the second most frequent cause of ADH (5 to 10% of the cases). Apolipoprotein B is the ligand of LDLR, and ADH-causative variants in *APOB* prevent or alter the binding of LDL particles to their receptor [12]. Less frequent (2% of the cases) are *PCSK9* gain-of-function mutations that are associated with high LDL-C levels due to increased lysosomal LDLR degradation [9,13]. The least frequent are mutations in *APOE* (1% of the cases) that also affect LDL binding to its receptor [14].

Besides the four major genes, ADH-causative defects are found in “minor genes” [5]. Recessive forms of hypercholesterolemia including autosomal recessive hypercholesterolemia (ARH) are linked to heterozygous variants in *LDLRAP1* which results in increased LDL-C levels [15]. Putative pathogenic variants in the adenosine triphosphate-binding cassette (*ABC)* transporter genes *G5* and *G8* that are involved in the recessive disease sitosterolemia (OMIM #210250) were found in 2.4% of subjects in an ADH Dutch cohort [16]. Possible pathogenic variants of the lysosomal acid lipase A gene (*LIPA)* that are involved in recessive deficiency of lysosomal acid lipase (LAL) (OMIM #278000) were found in 2.2% of subjects in an ADH Portuguese cohort [17]. In addition, a frameshift variant in

the gene encoding the enzyme cholesterol 7α-hydroxylase *(CYP7A1)* segregates with high LDL-C levels in a large family [18]. Cholesterol 7α-hydroxylasecatalyzes the rate-limiting step in the conversion of cholesterol to bile acids in the liver (OMIM #118455). Several rare missense variants in the gene encoding LDL receptor-related protein 6 (*LRP6)* which plays a crucial role in lipoprotein endocytosis and is an essential co-receptor in the Wnt/ß-catenin signaling pathway (OMIM #603507) [19,20] are linked to metabolic syndrome, high LDL-C levels, and early onset of CVD [21–23]. Rare or low-frequency variants in the gene encoding patatin-like phospholipase domain-containing 5 (*PNPLA5)* were significantly associated with LDL-C in an American cohort [24].

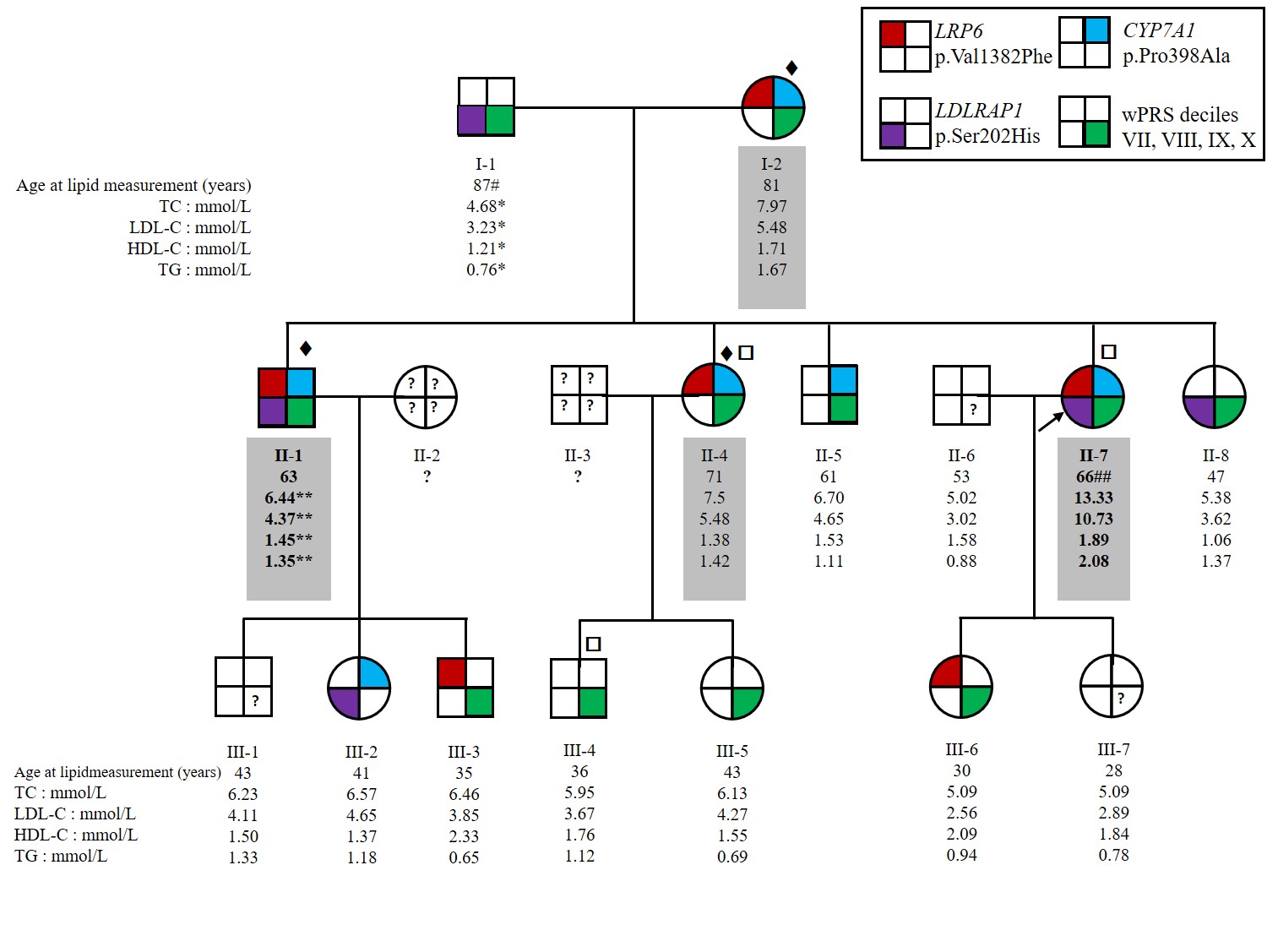
Mutations identified in genes causing ADH account for approximately 80% of cases [25]. Based on weighted polygenic risk score (wPRS) calculations [26], a polygenic origin may be suggested in 36% of nonmutated hypercholesterolemic patients [27]. This is in favor of the existence of a greater level of genetic heterogeneity in ADH and the involvement of unknown genes [28]. Between monogenic and polygenic forms of ADH, several digenic forms were reported with double-heterozygous carriers of mutations in two ADH major genes (*LDLR*/*APOB*, *LDLR*/*PCK9* [29] or *APOB*/*PCSK9* [30]) or in one major gene (*LDLR*, *APOB*, *PCSK9*, *APOE*) and one minor gene (*ABCG5*, *ABCG8*, *LDLRAP1*) [31]. To our knowledge, oligogenic forms with three variants in non-conventional ADH genes have not been reported.

We conducted this study in one French ADH family and in 160 unrelated hypercholesterolemic French probands in whom no pathogenic variant was found in the four ADH major genes. Our aim was to identify a new gene responsible for the ADH phenotype.

**2. Results**

2.1. Patient characteristics

The family HC438 recruited for this study was large enough to conduct genetic studies to identify new genes causing ADH. The index case for this family, II-7 (**Figure 1)**, had a total cholesterol level of 9.33 mmol/L and LDL-C of 6.67 mmol/L before lipid-lowering therapy at an age of 56 years. Her levels reached 13.33 mmol/L for total cholesterol and 10.73 mmol/L for LDL-C without treatment at age 66 years suggesting a homozygous form of ADH. She suffered from severe atheroma and had no extravascular cholesterol deposits. She also had a family history of hypercholesterolemia and cardiovascular heart disease. All willing members of the family were recruited resulting in an expansion to 15 individuals over three generations, including four affected (I-2, II-1, II-4 and II-7) and 11 normocholesterolemic (ie, normal cholesterol) individuals (I-1, II-5, II-6, and II-8, all third-generation) (**Figure 1**). Both parents of the index case II-7 had elevated LDL-C or CVD. The father, I-1, had a myocardial infarction at age 75 years and an LDL-C level of 3.23 mmol/L under ciprofibrate treatment at age 87 years. Because his LDL-C was normal, we considered him to be unaffected. We reasoned that he carried one or more variants that were not pathogenic singularly but became so in association with other variants. The mother, I-2, had an LDL-C level of 5.48 mmol/L under diet at age 81 years. The LDL-C level of subject II-1 at age 63 years was 4.37 mmol/L under 5 mg rosuvastatine treatment and was estimated to be 7.95 mmol/L without treatment [32]. Because of the absence of consanguinity in the family, patients II-1 and II-7 had higher LDL-C levels than their sister II-4. Based on these differences, II-1 and II-7 probably inherited two traits, one from each parent, whereas II-4 inherited the disease from only one parent (**Figure 1**). None of the affected family members presented extravascular cholesterol deposits or CVD, except subjects I-1 and II-7 which had CVD.



**Figure 1: Pedigree of the family HC438 with the segregation of p.(Val1382Phe) variant in *LRP6*, p.(Pro398Ala) variant in *CYP7A1* and p.(Ser202His) variant in *LDLRAP1* and the weighted Polygenic Risk Score (wPRS).** The proband II-7 is indicated by the black arrow. Squares and circles represent men and women, respectively.Affected family members are indicated by values highlighted in grey. More severely affected family members are indicated by bold values.

# Myocardial infarction at 75 years old, ## severe atheroma. \* under ciprofibrate, \*\* under rosuvastatin 5.Patients for whom ♦ whole exome,  whole genome sequencing was performed.TC: total cholesterol; LDL-C: LDL-cholesterol; HDL-C: HDL-cholesterol; TG: triglycerides.

In the cohort of 160 non-LDLR/non-APOB/non-PCSK9/non-APOE probands, lipid values before treatment were available for 98 patients (62.24% women, age 52.5±19 years): 7.96±1.76 mmol/L for total cholesterol, 5.9±1.24 mmol/L for LDL-C, 1.58±0.71 mmol/L for HDL-C and 1.57±1.1 mmol/L for triglycerides. The wPRS was calculated and 55.2% of the probands were in the top four deciles (VII, VIII, IX and X) of the WHII reference cohort (**Figure S1**).

2.2. Identification of candidate variants by whole genome sequencing, whole exome sequencing and positional cloning

We conducted whole genome sequencing (WGS) and whole exome sequencing (WES) of family HC438 and the data was analyzed conjointly. Using a dedicated in-house Python pipeline, 22 variants within the family meeting the filtering criteria were identified in the heterozygous state. Four variants were transmitted from the mother (I-2) to the three affected children (II-1, II-4 and II-7) corresponding to three missense variants in CYP7A1, KIFC2 and LRP6 and one intronic variant in SLC39A4. Three missense variants in the LDLRAP1, GOLGA4 and AP2A1 were transmitted from the father (I-1) to the two more severely affected children (II-1 and II-7). Three variants were transmitted from the mother (I-2) to the two more severely affected children (II-1 and II-7) corresponding to one missense variant in MOGAT2 and two intronic variants in PEX19 and TSC2. Finally, 12 UTR variants were transmitted by either parent to the three affected children (II-1, II-4 and II-7) (**Table S1**).

We performed positional cloning using a genome-wide scan of the entire family with 1072 polymorphic microsatellite markers. Parametric linkage analyses were performed under four hypotheses: 1) a paternal trait inherited by the three affected children, 2) a paternal trait inherited by the two more severely affected children, 3) a maternal trait inherited by the three affected children, and 4) a maternal trait inherited by the two more severely affected children (**Table S2**). The linkage analysis showed a maximum expected logarithum of odds score (ELOD) between 1.61 and 2.00 for the HC438 family which only allowed significant exclusion of the rs62371472 variant in the AP3S1 gene and the probable exclusion of the rs541351955 variant in the SMAP2 gene from paternal and maternal inheritance. The variants CD59 3’UTR, LDLRAP1 p.(Ser202His) and GOLGA4 p.(Arg1494Ile) were probably linked by paternal inheritance. The variants CYP7A1 p.(Pro398Ala), LRP6 p.(Val1382Phe) and SLC2A3 3’UTR as well as TSC2 intronic variants were probably linked by maternal inheritance. These seven genes with nonsynonymous variants were probably linked to a paternally or maternally inherited trait.

Among the seven genes, we selected the three variants in the ADH minor genes CYP7A1, LRP6 and LDLRAP1 for further analysis. We performed segregation analysis by Sanger sequencing of all recruited family members. The variants p.(Val1382Phe) in LRP6 and p.(Pro398Ala) in CYP7A1 were transmitted from the mother I-2 to the three affected children II-1, II-4 and II-7. The variants were also transmitted to the two unaffected members III-3 and III-6 for the LRP6 variant and II-8 and III-2 for CYP7A1 in favor of incomplete penetrance of the associated phenotypes (**Figure 1**). The p.(Ser202His) variant in LDLRAP1 was transmitted from the father I-1 to the two more severely affected children II-1, II-7; however, it was also transmitted to two unaffected members (II-8 and III-2) (**Figure 1**). Thus, the two more severely affected family members II-1 and II-7 carried the three variants. In silico analysis of these variants is detailed in **Table 1**.

**Table 1.** **Variants in *CYP7A1*, *LDLRAP1* and *LRP6*.** The pathogenicity of the variants was evaluated using Varsome, PolyPhen2, Provean, ClinVar, CADD score, and Splice AI.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Gene** | **c.notation**  **p.notation** | **rs number** | **Pathway** | **GTEx-TPM\_Liver #** | **gnomAD**  **(total)\*** | **gnomAD (ENF)\*** | **FREX\*\*** | **Varsome\*\*\*** | **PolyPhen2** | **Provean †** | **ClinVar** | **CADD Score ‡** | **Splice AI** |
| *CYP7A1*  (NM\_000780) | c.38C>T  p.(Ala13Val) | rs147162838 | Bile acid and bile salt metabolism | 2.612 | 0.181%  (512/282726) | 0.35%  (451/129062) |  | LB | B | N  (-0.189) | LB/VUS | 7.125 | No-consq  (0) |
| c.1039G>A  p.(Asp347Asn) | rs8192875 | 0.274%  (776/282802) | 0.019%  (25/129150) | … | LB | PD | D  (-0.2990) | … | 33 | Donor gain (0.48) |
| c.1192C>G  p.(Pro398Ala) | rs142708991 | 0.336%  (951/282868) | 0.43%  (555/129184) | 0.0871% | LB | PD | D  (-7.559) | LB | 25.1 | No-consq  (0) |
| *LDLRAP1*  (NM\_015627) | c.603dupC  p.(Ser202LeufsTer19) | rs781585299 | Clathrin-mediated endocytosis | 112.23 | … | … | … | P | … | … | P | … | … |
| c.604\_605delTCinsCA  p.(Ser202His) | rs386629678 | … | … | … | LB | PD | N  (-2.072) | LB | … | … |
| *LRP6*  (NM\_002336) | c.2915A>G  p.(Tyr972Cys) | rs772441071 | Vesicle-mediated transport | 9.662 | 0.001193%  (3/251364) | 0.002640%  (3/113656) | … | VUS | PD | D  (-7.227) | … | 26.9 | Acceptor gain (0.04) |
| c.4144G>T  p.(Val1382Phe) | rs139480047 | 0.08379%  (237/282856) | 0.1061%  (137/129164) | 0.261% | B | B | N  (-1.246) | LB | 22.3 | Donor gain (0.02) |
| c.4436C>T  p.(Thr1479Ile) | rs144175121 | 0.02263%  (64/282836) | 0.04335%  (56/129168) | … | B | B | N  (-1.906) | … | 23.3 | No-consq  (0) |
| c.4835C>T  p.(Ser1612Phe) | … | 0.0008097%  (2/247016) | 0%  (0/111624) | … | VUS | PD | D  (-2.879) | … | 29.4 | Donor gain (0.02) |

# Gene expression in the liver, from the Genotype Tissue Expression database (GTEx). TPM: transcripts per million

\* Allele frequency, from the Genome Aggregation Database (gnomAD): allele count/allele number in the general population and the European non-Finnish (ENF)

\*\* Allele frequency from the French Exome Project database.

\*\*\* Varsome tool according to the ACMG guidelines [51]

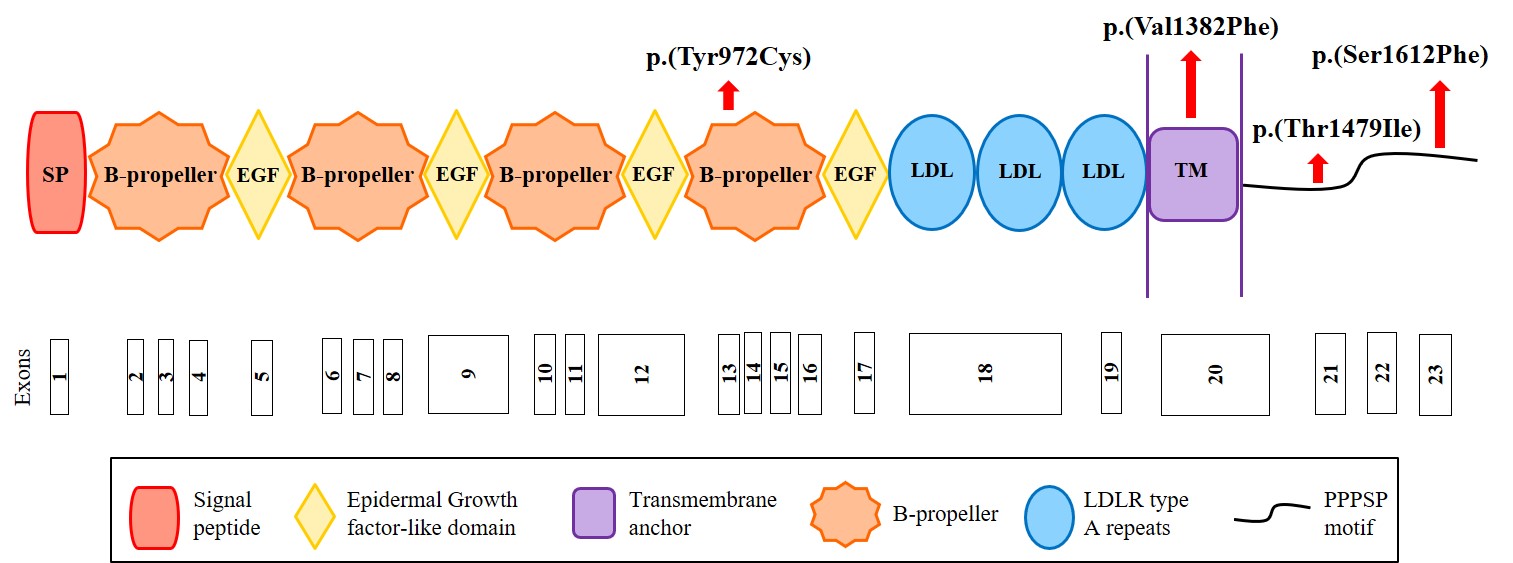
**†** Provean:Variant with a score ≤-2.5 is considered “deleterious” and with a score > -2.5 is considered “neutral”.

**‡** CADD score ≥ 20 indicates that the variant is predicted to be among the top 1% of the most deleterious substitutions in the human genome, and a score ≥ 30 indicates that the variant is predicted to be among the top 0.1% of the most deleterious substitutions in the human genome.

N: neutral, LB: likely benign, B: benign, VUS: variant of unknown significance, PD: probably damaging, D: deleterious, P: pathogenic

2.3. Molecular studies of CYP7A1, LDLRAP1 and LRP6

We sequenced the CYP7A1, LDLRAP1 and LRP6 genes in the cohort of 160 French hypercholesterolemic probands in whom mutations in LDLR, APOB, PCSK9, and APOE had been excluded. We replicated the CYP7A1 finding by identifying the same p.(Pro398Ala) mutation in a 71-year-old man who presented total cholesterol of 7.52 mmol/L and LDL-C of 5.69 mmol/L. Among other cohort members, we identified the two variants in this gene and the four rare missense variants p.(Tyr972Cys), p.(Thr1479Ile) and p.(Ser1612Phe) in LRP6 and p.(Ser202LeufsTer19) in LDLRAP1 (**Tables 1 and 2**). The p.(Tyr972Cys) variation in LRP6 was located in the fourth ß-propeller domain of the LRP6 receptor, whereas two other variants were in the intracellular domain (**Figure 2**). The substituted residues were highly conserved among species from human to zebrafish (**Figure S1**). The p.(Val1382Phe) variant in LRP6 was located in the transmembrane domain of LRP6 (**Figure 2**) in a conserved region (**Figure S2**).



**Figure 2. Structure of the LRP6 receptor and position of the identified variants.** LRP6 receptor contains the following structural motifs: signal peptide (SP), four β-propeller domains, four EGF like domains (involved in the pH-dependent release of ligands in endosome), three LDLR type A repeats (responsible for the binding of ligands), a transmembrane anchor (binds the receptor to the cell membrane), and a cytoplasmic domain with PPPSP motifs (two motifs at position 1487 and 1604 that allow the receptor to function in the Wnt/β-catenin pathway). Red arrows indicate the position of the variants identified in this study.

Figure built from data from UniProt ([www.uniprot.org](http://www.uniprot.org/)) and Ensembl ([www.ensembl.org/index.html](http://www.ensembl.org/index.html)) databases.

**Table 2.** **Biological and Clinical characteristics of the affected carriers of *CYP7A1, LDLRAP1* and *LRP6* variant**

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Gene** | **Variant** | **Sex** | **Age \*** | **TC \*\*** | **LDL-C \*\*** | **HDL-C \*\*** | **TG \*\*** | **wPRS** | **Decile** | **Clinic** | **Family History** |
| *CYP7A1*  (NM\_000780) | c.38C>T  p.(Ala13Val) | M | 59 | … | … | … | … | 0.571 | IV | … | … |
| c.1039G>A  p.(Asp347Asn) | F | 71 | 7.92 | 6.24 | … | 0.98 | 0.25 | I | … | … |
| c.1192C>G  p.(Pro398Ala) | M | 71 | 7.52 | 5.69 | … | … | … | … | … | … |
| *LDLRAP1*  (NM\_015627) | c.603dupC  p.(Ser202LeufsTer19) | F | 37 | 7.75 | 5.56 | 1.37 | 1.81 | 0.752 | VII | … | … |
| c.604\_605delTCinsCA  p.(Ser202His) | F | 39 | … | 5.44 | … | … | 0.622 | V | … | … |
| *LRP6*  (NM\_002336) | c.2915A>G  p.(Tyr972Cys) | M **†** | 40 | 6.35 | 4.70 | 1.21 | 0.89 | … | … | CAD | Yes  Yes |
| M  **‡** | 32 | 7.47 | 5.30 | 1.34 | 1.83 | 0.371 | II | No |
| c.4436C>T  p.(Thr1479Ile) | F | 48 | 8.15 | 5.57 | 2.12 | 1.01 | 0.581 | IV | No | Yes |
| c.4835C>T  p.(Ser1612Phe) | M | 51 | 8.04 | 5.89 | 1.71 | 0.94 | 0.542 | III | CAD | Yes |

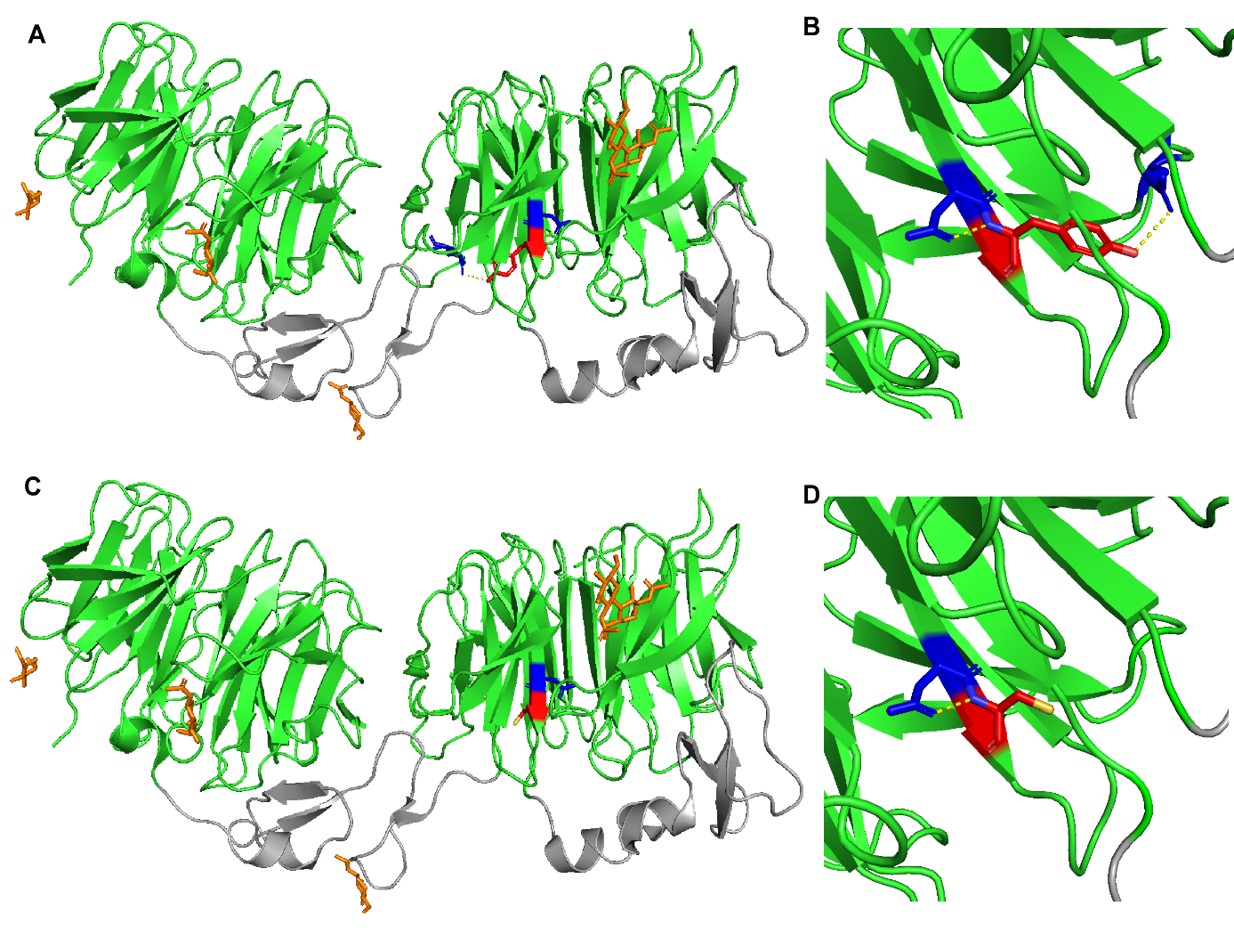
\* Age in years at lipid measurement

**\*\*** Lipid values (mmol/L) without lipid-lowering therapy

† Father ‡ Son

TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; TG: triglycerides; wPRS: weighted polygenic risk score.

The use of PyMOL to model the effects of the p.(Tyr972Cys) in LRP6-E3E4 (PMB ID 3S8Z) showed that this missense variant resulted in the loss of polar interaction with a neighboring glutamic acid residue at position 993 (**Figure 3 A-D**). As a result, it is predicted that this mutant could result in changes in the stability of LRP6 or its ability to uptake LDL. A crystal structure of the transmembrane and intracellular domains of LRP6 containing its transmembrane and intracellular domains was not available so the variants p.(Val1382Phe), p.(Thr1479Ile), p.(Tyr1584Asn), and p.(Ser1612Phe) could not be modeled.



**Figure 3. Crystal structure of wild-type and mutant LRP6-E3E4 with β-propeller domains (green) and epidermal growth factor (EFG)-like domains (grey)**. **(A)** and **(B)** LRP6-E3E4 Tyr972 residue (red) has polar contacts with Asp971 and Glu993 (blue). **(C)** and **(D)** LRP6-E3E4 mutant Cys972 residue (red) has polar contact only with Asp971 (blue).

2.4. Bile acid synthesis is not affected by the CYP7A1 variant p.(Pro398Ala)

Cholestanols including 7α-hydroxy-cholesterol levels were significantly lower in carriers of p.(Pro398Ala) in CYP7A1 compared to non-carriers in the HC438 family (p = 0.015, p = 0.032, respectively), whereas bile acid levels were not affected (**Table 3**). This variant, therefore, participated in the elevation of LDL-C levels. However, this cannot fully explain the phenotype because family members II-5 and III-2 carrying this variant did not present hypercholesterolemia.

**Table 3.** **Sterol and bile acid measurements in family HC438**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **II-1 †\*** | **II-4 †\*\*** | **II-5 †** | **III-4** | **III-6** | **III-7** | **Carriers \*\*\*** | **Non-carriers \*\*\*** | **Marker of** | **P value** |
| Serum total cholesterol (mmol/L) | 5.08 | 7.84 | 6.95 | 5.73 | 5.46 | 3.67 | 6.62 ± 1.41 | 4.95 ± 1.12 |  | 0.306 |
| R\_campesterol (μg/mg) **‡** | 1.77 | 1.13 | 1.41 | 1.66 | 1.98 | 2.02 | 1.44 ± 0.32 | 0.32 ± 1.89 | cholesterol absorption | 0.061 |
| R\_sitosterol (μg/mg) **‡** | 1.65 | 0.87 | 0.97 | 0.94 | 1.26 | 1.30 | 1.16 ± 0.43 | 1.17 ± 0.20 | cholesterol absorption | 0.495 |
| R\_cholestanol (μg/mg) **‡** | 1.05 | 1.00 | 0.79 | 1.32 | 1.25 | 1.38 | **0.95 ± 0.14** | **1.32 ± 0.07** | cholesterol absorption | **0.015** |
| R\_lathosterol (μg/mg) **‡** | 0.68 | 1.46 | 1.39 | 2.02 | 1.22 | 2.01 | 1.18 ± 0.43 | 1.75 ± 0.46 | cholesterol synthesis | 0.094 |
| R\_lanosterol (µg/mg) **‡** | 0.10 | 0.14 | 0.12 | 0.17 | 0.11 | 0.20 | 0.12 ± 0.02 | 0.16 ± 0.05 | cholesterol synthesis | 0.126 |
| R\_desmosterol (μg/mg) **‡** | 0.65 | 0.78 | 0.77 | 0.91 | 0.58 | 0.77 | 0.74 ± 0.07 | 0.75 ± 0.17 | cholesterol synthesis | 0.433 |
| R\_7αOH-cholesterol (ng/mg) **‡** | 23 | 25 | 39 | 41 | 44 | 52 | **29 ± 9** | **46 ± 6** | degradation to bile acids | **0.032** |
| R\_27OH-cholesterol (ng/mg) **‡** | 113 | 108 | 95 | 107 | 117 | 128 | 105 ± 9 | 118 ± 10 | degradation to bile acids | 0.104 |
| Chenodeoxycholic acid (μmol/L) | 3.05 | 2.76 | 1.34 | 2.11 | 0.01 | 3.49 | 2.38 ± 0.91 | 1.87 ± 1.75 | bile acids | 0.342 |
| Cholic acid (µmol/L) | 1.76 | 1.92 | 0.44 | 1.74 | 0.01 | 1.28 | 1.38 ± 0.81 | 1.01 ± 0.89 | bile acids | 0.314 |
| Lithocholic acid (µmol/L) | 0.06 | 0.16 | 0.21 | 0.20 | 0.31 | 0.11 | 0.14 ± 0.08 | 0.21 ± 0.10 | bile acids | 0.224 |
| Deoxycholic acid (µmol/L) | 0.27 | 2.16 | 2.30 | 2.99 | 1.95 | 1.06 | 1.58 ± 1.13 | 2.00 ± 0.96 | bile acids | 0.324 |

† carriers of the variant p.(Pro398Ala) in *CYP7A1*

\* under rosuvastatine 5 mg

\*\* under pravastatine 20 mg

‡ values corrected for cholesterol concentration (R\_sterols)

\*\*\* Mean ± SD

2.5. Contradictory effects of LRP6 variants on LRP6 expression and LDL binding and uptake

HuH7 cells transfected with a siRNA targeting human LRP6 (siLRP6) showed the expected reduced expression of the LRP6 gene by approximately 80% (**Figure 4A**). No significant differences in LDLR, HMGCR, SREBP2, and PCSK9 mRNA expression were observed (**Figure S3**), whereas labeled LDL uptake was significantly reduced by 23.14% and 20% compared to non-transfected cells and to cells transfected with the negative control (siNeg), respectively (p<0.05) (**Figure 4B**). As expected, cells transfected with the LRP6-WT plasmid presented a significant increase in membrane expression of LRP6 compared to cells transfected with the empty vector (PcM) (**Figure 4C**), but there was no significant difference in the binding and uptake of labeled LDL (**Figure 4F** and **Figure S4**).

The two variants, p.(Tyr972Cys) and p.(Thr1479Ile) significantly decreased surface expression of LRP6 compared to WT (p<0.001) (**Figure 4C**), but only p.(Thr1479Ile) significantly decreased LRP6 expression in cells (**Figure 4D-E**). The two variants increased the binding and uptake of labeled LDL in HEK293T cells. However, a significant difference (p<0.05) was observed only with the p.(Tyr972Cys) variant compared to LRP6-WT for binding and uptake measured simultaneously (**Figure S4**) and for uptake alone **(Figure 4F)**.

Unlike p.(Tyr972Cys) and p.(Thr1479Ile), p.(Val1382Phe) did not affect membrane expression of LRP6 (**Figure 4C**), but significantly it reduced LDL uptake when compared to LRP6-WT (p<0.05) **(Figure 4F).** Nevertheless, this variant had no significant effectwhen LDL binding and uptake were observed simultaneously (**Figure S4)**. The effect of LRP6-WT or the mutated plasmids on membrane expression of the LDL receptor was also evaluated in transfected HEK293T cells and no significant changes were observed (**Figure S5**).

Chart, diagram

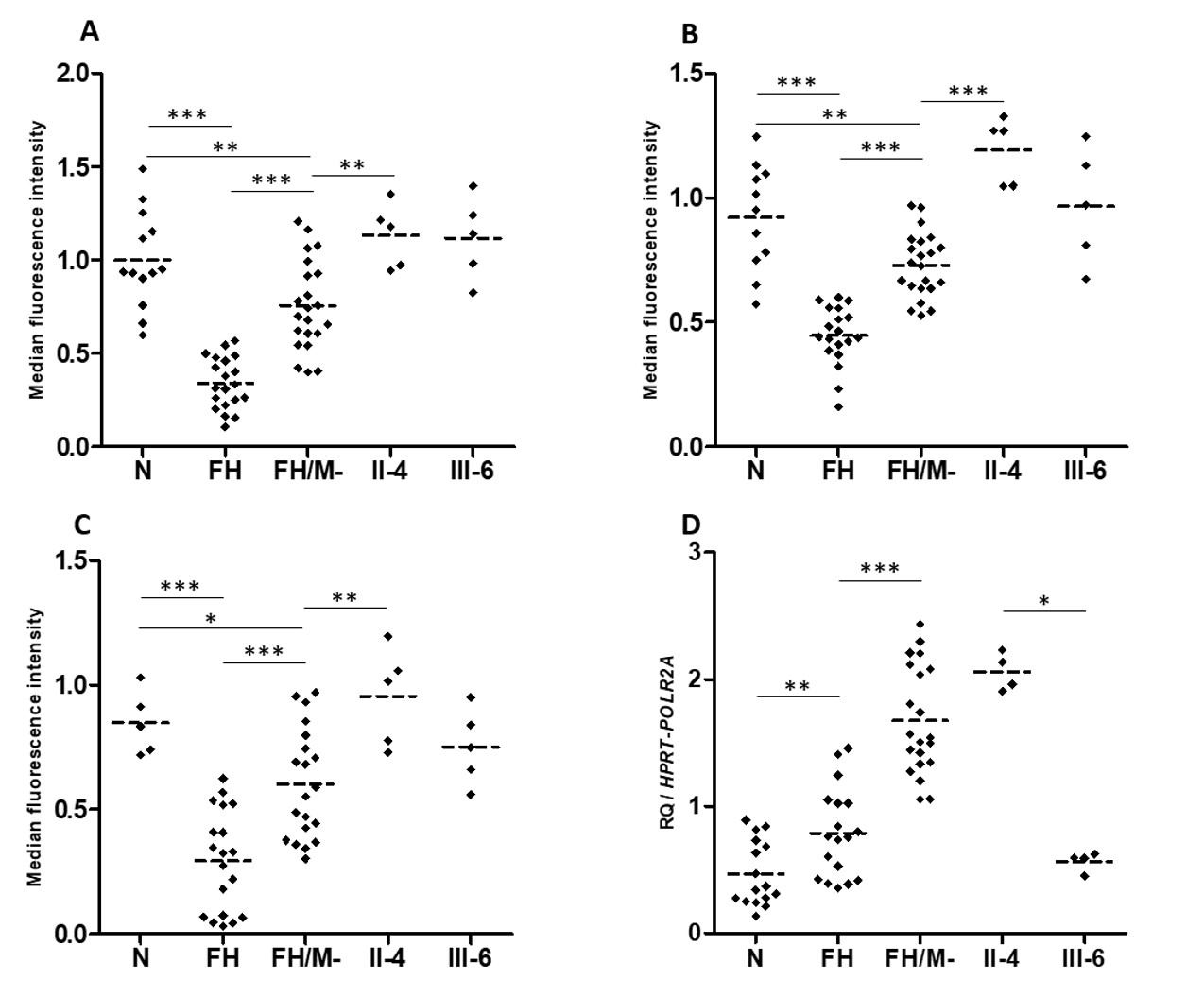
Description automatically generated

**Figure 4. Effect of inhibited, overexpressed or mutated *LRP6* in HEK293T and HuH7 cells.** (**A**) ***LRP6* mRNA expression in HuH7 after silencing of *LRP6*.** Reactions were run in triplicate for each cDNA. *POLR2A* was used as the reference housekeeping gene. The relative quantification of gene expression was performed using the ∆∆CT method and non-transfected cells were used for calibration. (**B**) **LDL-Bodipy uptake in HuH7 after silencing of *LRP6*.** Median fluorescence intensity of 50000 events was acquired for each sample but only the median fluorescence intensity of living cells is presented. Data represent three independent assays performed in triplicate. (**C**) **Expression of WT or mutated LRP6 at the cell surface of transfected HEK293T**. The median fluorescence intensity of 100000 events was acquired for each sample but only the median fluorescence intensity of living cells is presented. Data represent four independently performed assays. (**D**) and (**E**) **LRP6 expression in HEK293T cells after transfection with LRP6-WT or mutated plasmids (p.(Thr1479Ile) and p.(Tyr972Cys) variants)**. Proteins were extracted from transfected cells, separated by electrophoresis then transferred to PVDF membrane. The membrane was incubated with primary antibody (anti-LRP6) followed by incubation with secondary antibody before detection using the iBrightTM FL1500 imaging system. Protein was quantified by ImageJ software. Equal loading was confirmed using ß-actin antibody. Data represent three independent assays. (**F**) **LDL uptake in HEK293T after transfection with empty vector, LRP6-WT or mutated plasmid.** The median fluorescence intensity of 100000 events was acquired for each sample but only the median fluorescence intensity of living cells is presented. The fluorescence of each sample was normalized using the empty vector (PcM) as reference. Data represent three independent assays each performed in triplicate.

In all experiments, the difference between conditions was determined by Bonferroni's Multiple Comparison Test in one-way ANOVA. \* p<0,05, \*\* p<0,01 and \*\*\* p<0,001 were considered as statistically significant. Results are shown as mean±SD. Error bars represent ± SD.

2.6. EBV-transformed B-lymphocytes from heterozygotes carriers of p.(Val1382Phe) did not present altered LDL uptake or LRP6 gene expression

The number of LDL cell surface receptors, LDL binding or LDL uptake was similar for EBV-transformed B-lymphocytes from two heterozygotes carriers of LRP6-p.(Val1382Phe) compared to cells from normocholesterolemic subjects. However, these parameters were significantly reduced in B-lymphocytes from the familial hypercholesterolemia (FH) patients (**Figure 5A-C**). Interestingly, LRP6 gene expression is significantly higher in EBV-transformed B-lymphocytes from FH patients compared to cells from normocholesterolemic subjects (**Figure 5D**). Cells from affected patient II-4 carrying p.(Val1382Phe) presented expression similar to that of LRP6 in cells from FH patients, whereas cells from unaffected carrier (III-6) presented expression of LRP6 similar to that of cells from normocholesterolemic subjects (**Figure 5D**).



**Figure 5. LDL receptor expression, LDL binding and uptake, and LRP6 gene expression in patient EBV-transformed B-lymphocytes.** (A) LDL receptor, (B) LDL-Bodipy binding and (C) LDL-Bodipy uptake quantification in EBV-transformed B-lymphocytes from normocholesterolemic subjects (N), LDLR mutation carriers (FH), hypercholesterolemic patients without an identified mutation (FH/M-), and two LRP6-p.(Val1382Phe) carriers from the HC438 family: II-4 and III-6 (see Figure 1). The median fluorescence of living cells is presented. Data represent five independently performed assays. (D) LRP6 gene expression. Relative Quantification (RQ) of LRP6 in EBV-transformed B-lymphocytes. Reactions were run in triplicate for each cDNA. HPRT and POL2RA were used as reference genes. The relative quantification was performed using the ∆CT method.

(A-D). Bonferroni's Multiple Comparison Test in 1 way ANOVA: \* p<0,05, \*\* p<0,01, \*\*\* p<0,001.

2.7. Carriers of a LRP6 or LDLRAP1 variant present lower LDL-C levels than non-carriers despite a similar polygenic risk score

We used a wPRS that includes risk alleles from the six frequent LDL-C-associated genes CELSR2, APOB, ABCG5/8, LDLR, and APOE [26]. As expected, the mean LDL-C levels were significantly higher in the 152 ADH probands compared to the 13 non-ADH subjects (6.1 ± 1.5 vs 3.4 ± 0.5 mmol/L, p < 0.0001) (**Table 4).** The mean LDL-C levels in the four carriers of CYP7A1 variants (4.6 ± 1.3 vs 3.4 ± 0.5 mmol/L, p = 0.0242) and the six carriers of LRP6 variants (5.5 ± 0.8 vs 3.4 ± 0.5 mmol/L, p = 0.0053) were significantly higher compared those of the 13 non-ADH subjects (**Table 4)**. The mean LDL-C levels in the 152 ADH probands were also significantly higher than those of the six carriers of LRP6 variants (4.6 ± 1.3 vs 6.1 ± 1.5 mmol/L, p=0.0088) (**Table 4**). These LDL-C differences were not attributable to a polygenic contribution because the wPRS were statistically similar among the ADH probands and the three variant genes (**Table 4**). Nevertheless, with two to six subjects in the gene variant groups, our results can only be considered as a simple observation and must be confirmed by the study of larger groups.

In the HC438 family, all the affected members present a wPRS in the top four deciles (VII, VIII, IX, and X) of the WHII reference cohort as well as six unaffected members (**Figure 1**). The wPRS does not influence the LDL-C levels in this family.

**Table 4.** **LDL-C levels and weighted Polygenic Risk Score (wPRS) comparison among the carriers of *CYP7A1*, *LRP6* and/or *LDLRAP1* variants.**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Non-ADH subjects\*** | **ADH Probands\*\*** | ***CYP7A1* variant carriers\*\*\*** | ***LRP6* variant carriers#** | ***LDLRAP1* variant carriers##** | ***CYP7A1* and *LRP6* variants carriers###** | ***CYP7A1*, *LRP6* and *LDLRAP1* variants carriers†** |
| **N** | 13 | 152 | 4 | 6 | 3 | 2 | 2 |
| **Sexe (% of women)** | 53.8 | 60.4 | 25 | 33.3 | 66.7 | 100 | 50 |
| **Age (years)** | 50 ± 15 | 48 ± 18 | 66 ± 6 | 39 ± 9 | 54 ± 28 | 81 - 71 | 63 - 66 |
| *p value vs non-ADH subjects* | | *0.4897* | ***0.0266*** | *0.0520* | *0.3185* |  |  |
| *p value vs ADH probands* | |  | ***0.0094*** | ***0.0453*** | *0.4945* |  |  |
| **LDL-C (mmol/L)** | 3.4 ± 0.5 | 6.1 ± 1.5 | 5.5 ± 0.8 | 4.6 ± 1.3 | 4.7 ± 1.3 | 5.5 – 5.5 | 7.9 – 10.7 |
| *p value vs non-ADH subjects* | | ***<0.0001*** | ***0.0053*** | ***0.0242*** | *0.0693* |  |  |
| *p value vs ADH probands* | |  | *0.2364* | ***0.0088*** | *0.0542* |  |  |
| **wPRS** | 0.665 ± 0.165 | 0.700 ± 0.187 | 0.574 ± 0.326 | 0.655 ± 0.243 | 0.792 ± 0.193 | 0.731 - 0.831 | 0.831 - 0.902 |
| *p value vs non-ADH subjects* | | *0.2024* | *0.3428* | *0.500* | *0.2091* |  |  |
| *p value vs ADH probands* | |  | *0.2091* | *0.2548* | *0.2676* |  |  |

\* Normocholesterolemic and Non-carriers of a rare variant in ADH-causing genes.

\*\*Non-carriers of a rare variant in *LDLR*, *APOB*, *APOE*, *PCSK9*, *CYP7A1*, *LRP6*, *LDLRAP1* genes.

\*\*\* One p.(Ala13Val), one p.(Asp347Asn), and two p.(Pro398Ala) (**Figure 1, Table 2**).

# Two p.(Tyr972Cys), two p.(Val1382Phe), one p.(Thr1479Ile), and one p.(Ser1612Phe) (**Figure 1, Table 2**).

## One p.(Ser202LeufsTer19) and two p.(Ser202His) (**Figure 1, Table 2**).

### Two p.(Pro398Ala) in *CYP7A1* and p.(Val1382Phe) in *LRP6* (**Figure 1**).

**†** Two p.(Pro398Ala) in *CYP7A1*, p.(Val1382Phe) in *LRP6,* and p.(Ser202His) in *LDLRAP1* (**Figure 1**).

3. Discussion

We report here the first joint analysis of WES and WGS to identify ADH-causing genes in a French family. WES was performed for the three family members I-2, II-1 and II-4, and WGS was performed for II-4 and the two additional family members II-6 and III-3. This permitted the analysis of five affected and unaffected members over three generations. WGS was used to complete WES with better genome coverage. The joint analysis permitted the filtering of variants according to segregation over the three generations with retention of information and better accuracy compared to separate analyses of the two sequencing results.

This approach allowed us to identify ADH proband II-7 (**Figure 1)** who had no detectable causal mutation in the four major ADH genes. II-7 carried the three rare variants (p.(Pro398Ala) in *CYP7A1*, p.(Val1382Phe) in *LRP6* and p.(Ser202His) in *LDLRAP1* in a polygenic background. The genotyping of these three variants with the wPRS calculation in the whole proband family allowed the identification of affected member II-1 with the same oligogenic/polygenic combination as well as the two affected members I-2 and II-4 with the CYP7A1/LRP6/polygenic combination. None of the family members presenting a high polygenic score alone or carrying one of the variants was affected (**Figure 1)**. To identify ADH carriers with oligogenic combinations, we sequenced the three minor genes *CYP7A1*, *LRP6* and *LDLRAP1*. We also sequenced 160 unrelated ADH probands with no causal variants in the four major genes resulting in six additional rare variants. The two variants p.(Ala13Val) and p.(Asp347Asn) were found in *CYP7A1,* the three variants p.(Tyr972Cys), p.(Thr1479Ile) and p.(Ser1612Phe) were found in *LRP6* and the single variant p.(Ser202LeufsTer19 was found in *LDLRAP1* (**Table 1 and 2**). No oligogenic combination of these variants was identified in the same proband. We then evaluated the effect of the p.(Pro398Ala) in *CYP7A1* on bile acid synthesis and observed no major effect which would indicate a CYP7A1 deficiency (**Table 3**).Similarly, functional analyses of the four *LRP6* variants showed no major effect on LDL uptake (**Figures 4 and 5**). This suggests each variant alone is insufficient to reveal disease. Rather, a combination of variants is necessary.

The family proband II-7 had severe hypercholesterolemia suggesting a homozygous form of ADH (**Figure 1**) and thus inherited defects from both parents. Whole genome and exome sequencing complemented by positional cloning analysis revealed the three rare variants transmitted from the father and the four variants , from the mother (**Tables S1 and S2**). We selected the variants in the three ADH minor genes *CYP7A1*, *LRP6* and *LDLRAP1* for further analysis in the whole family and could not show good segregation with ADH for any individual. Indeed, each of the three variants was also carried by the unaffected family members II-8 and III-2 for p.(Pro398Ala) in *CYP7A1*, III-3 and III-6 for p.(Val1382Phe) in *LRP6* and I-1, II-8 and III-2 for p.(Ser202His) in *LDLRAP1* (**Figure 1**). Only the *CYP7A1* and *LRP6* variants were carried by the four affected family members, whereas the variants in *LDLRAP1* were present only in the two more severely affected children II-1 and II-7 (**Figure 1**). This suggests that the combined effects of the p.(Pro398Ala) in *CYP7A1* and p.(Val1382Phe) in *LRP6* are needed to reveal the ADH phenotype. Furthermore, ADH becomes more severe with the addition of p.(Ser202His) in *LDLRAP1*. Except for the unaffected member III-2, all family members presented a wPRS within the top 4 deciles of the WHII reference cohort and thus did not segregate with the ADH phenotype (**Figure 1**).

While carrying the same genotype and being the more severely affected subjects II-1 and II-7 had different LDL-C levels without treatment (7.95 vs 10.73 mmol/L). Phenotypic variability in ADH is frequently reported as the consequence of metabolic, environmental and genetic factors [3]. Evaluation of the eating behavior and lifestyle of these two patients as well as a search for modifying genetic factors will help to understand this difference. However, the higher wPRS for subject II-7 (0.902 in decile X) compared to II-1 (0.831 in decile IX) may partially explain this phenotypic difference. The *CYP7A1* gene encodes cholesterol 7 a-hydroxylase which is a 504 amino acid microsomal cytochrome P450 that catalyzes the rate-limiting reaction in the cholesterol catabolic pathway in the liver. This is the first step in the conversion of cholesterol to bile acids [33,34], and a deficiency of CYP7A1 would decrease bile acid production and accumulation of cholesterol in the liver. This would lead to retention of Sterol Regulatory Element Binding Protein (SREBP) in the endoplasmic reticulum, downregulation of LDL receptors, and consequent hypercholesterolemia. Several polymorphisms in *CYP7A1* are associated with LDL-C levels [35,36]. Markers for cholesterol absorption, synthesis and degradation to bile acids were measured in the serum of six members of the family. The results showed that a marker of cholesterol absorption (cholestanol) and a marker of cholesterol degradation to bile acids (7 α-hydroxy-cholesterol) were significantly lower in carriers of the p.(Pro398Ala) in *CYP7A1* compared to non-carriers (p = 0.015, p = 0.032, respectively), whereas bile acids were not affected (**Table 3**). Thus, we conclude that p.(Pro398Ala) in *CYP7A1* can participate in the elevation of the LDL-C level; however, this variant cannot solely explain the ADH phenotype in the family.

The human *LRP6* gene produces a 1613 amino-acid protein **(Figure 2)** that is a member of the LDL receptor family which consists of transmembrane cell surface proteins involved in receptor-mediated endocytosis of specific ligands [6,37]. The LRP6 receptor has a unique structure and is crucial for lipoprotein endocytosis. It also functions as an essential co-receptor for the Wnt/ß-catenin signaling pathway [19,20]. LRP6 has pleiotropic effects with an important role in cell differentiation, proliferation and migration during embryonic development. Pathogenic variants in *LRP6* are also associated with diverse human diseases [38]. The role of LRP6 in LDL metabolism was studied mainly by Mani et al. who identified the p.(Arg611Cys) missense variant in a family of Asian origin [39]. In addition to LRP6-(Arg611Cys), common variations within *LRP6* are associated with modest elevations in serum LDL in the general population. The common variant rs10845493 in *LRP6* is associated with elevated LDL levels [40] and rs2302685 (p.(Val1062Ile) is associated with hypercholesterolemia [23]. The functional analysis of the four LRP6 variants identified in this study showed contradictory results.

We observed that the overexpression of *LRP6* in HEK293T cells had no significant effect on LDL uptake (**Figure 4F**). This result differed from that of a previous study showing that overexpression of *LRP6* in CHO-K1 cells results in increased LDL internalization [37]. The discrepancy may be explained by the different cell types, HEK293T versus CHO-K1. Alternatively, our results showed that the knockdown of *LRP6* in HuH7 cells reduced LDL uptake (**Figure 4A and 4B**). This suggests that physiological levels of LRP6 are sufficient for LDL uptake in HEK293T cells, and consequently the overexpression of LRP6 does not affect LDL uptake.

The two *LRP6* variants p.(Tyr972Cys) and p.(Thr1479Ile) significantly decreased membrane expression of LRP6 compared to WT **(Figure 4C)**. This is similar to the p.(Arg611Cys) variant in human lymphoblastoid cells [41] and CHO-K1 cells [37]. However, the decrease in LRP9 membrane expression did not affect the expression of the LDL receptor (**Figure S5**) unlike the NIH3T3 variant p.(Arg611Cys) in human lymphoblastoid cells [41]. We tested the effects of the p.(Arg611Cys) variant in HEK293T cells and also showed a slight but not significant decrease in membrane expression of LRP6 with no effect on the expression of the LDL receptor (data not shown). Moreover, p.(Tyr972Cys), which reduced membrane expression of LRP6, significantly increased LDL uptake compared to LRP6-WT in HEK293T cells (**Figures 4F and S4**). These results were supported by PyMOL modeling of LRP6 p.(Tyr972Cys) that predicted conformational changes or altered interactions of LRP6 due to loss of polar interaction with the neighboring Glu993 residue (**Figure 3B-E**). The effect of p.(Tyr972Cys) on LDL uptake may be due to LRP6 competition with the LDL receptor for uptake. The absence of LRP6 at the cell surface may increase LDL uptake by the LDL receptor. Nevertheless, this effect may not be major and sufficient to significantly reduce LDL levels *in vivo*.

The p.(Val1382Phe) variant is located in the transmembrane domain of the LRP6 receptor (**Figure 2**) and is probably unable to internalize after binding to LDL particles. However, the variant probably did not alter LDL binding or affect the membrane expression of LRP6 (**Figures 4F and S4**). Nevertheless, this effect may not be major and sufficient to significantly increase LDL levels *in vivo*.

Note that these newly identified variants in *LRP6* are not located in the same domain of the receptor and none is in the 2nd EGF domain which is specifically needed to induce the release of bound LDL at low pH in the endosome [41] or the 2nd ß-propeller domain where the previously described variants are associated with metabolic syndrome and CVD (**Figure 2**). The variant p.(Ser202His) in *LDLRAP1* was heterozygous in all carriers, and other heterozygous variants are known to increase LDL-C levels [15]. However, none of the family members carrying p.(Ser202His) alone presented elevated LDL-C levels, whereas the two unrelated probands with p.(Ser202His) and p.(Ser202LeufsTer19) presented elevated LDL-C levels (**Table 2**).

Altogether, our results suggest that in the HC438 family, the combined effects of p.(Pro398Ala) in *CYP7A1* and p.(Val1382Phe) in *LRP6* are needed to reveal the ADH phenotype which becomes more severe with the addition of the p.(Ser202His) in *LDLRAP1*. To our knowledge, no direct interactions between CYP7A1-related metabolism and the LRP6 or LDLRP1 pathways have been described. Functional studies show that when the LDL particle binds to its receptor, LRP6 forms a complex with the LDL receptor, LDLRAP1 and clathrin to initiate endocytosis of the LDL receptor/LDL complex [19,37]. Thus, LRP6 and LDLRAP1 are essential for an efficient LDL endocytosis of the LDL. Furthermore, p.(Ser202His) in *LDLRAP1* probably worsens the effect of p.(Val1382Phe) in *LRP6* which leads to the more severe phenotype observed with the two affected family members II-1 and II-7 (**Figure 1**) carrying the two variants.

Obviously, it would be interesting to functionally analyze both LRP6 and LDLRAP1. However, we have chosen to study the effect of each gene variant and performed the functional analyses of the four variants in *LRP6* alone. A future study will have to examine the functional analysis for both (Val1382Phe) in *LRP6* and (Ser202His) in *LDLRAP1*. It will be interesting in the future to evaluate the effect of variants in the *CYP7A1*, *LRP6* or *LDLRAP1* genes on LDL-C levels and phenotypic variability among ADH patients with a pathogenic variant in a major ADH gene.

In summary, in the HC438 family, we have identified three variants in three different ADH minor genes: p.(Pro398Ala) in *CYP7A1*, p.(Val1382Phe) in *LRP6* and p.(Ser202His) in *LDRAP1*. We have excluded a major effect of each variant alone as well as the involvement of the wPRS. Thus, an oligogenic form of hypercholesterolemia is probably present in this family and high levels of LDL-C could be caused by the cumulative effect of *LRP6* and *CYP7A1* variants which is aggravated by the *LDLRAP1* variant.

4. Materials and Methods

4.1. Probands and family recruitment

Probands and family members were all of non-Finnish European origin and recruited through the French Research Network on hypercholesterolemia that includes 14 different lipid clinics in France. Affected probands and family members meet the following inclusion criteria: total and LDL-C above the 90th percentile when compared with a sex and age-matched French population (STANISLAS cohort [42]), normal levels of triglycerides and HDL-C, and autosomal dominant transmission of hypercholesterolemia in the family. Exclusion criteria included any diseases leading to secondary hypercholesterolemia. Lipid levels before initiation of treatment were used when available. For all subjects, the four ADH-causing genes were studied as previously reported [30]. This allowed us to identify probands in whom mutations in *LDLR*, *APOB*, *PCSK9,* and *APOE* had been excluded.Thus, the study population consisted of 160 non-*LDLR*/non-*APOB*/non-*PCSK9*/non-*APOE* probands and one family (HC438).

DNA analyses in human subjects were performed after informed consent was obtained from all subjects in agreement with French bioethics laws. The research project received IRB approval (research project trial #05-07-06) by the French Consultative Committee for the Protection of Person in Biomedical Research, Paris, Necker.

4.2. Whole genome and exome sequencing and data analysis

Whole exome sequencing (WES) was performed at the Broad Institute of Harvard and MIT (Cambridge, MA, USA) for the three affected members I-2, II-1, and II-4 from the HC438 family (**Figure 1**) as described previously [43]. Whole genome sequencing (WGS) was performed at the Centre National de Recherche en Génomique Humaine (CNRGH, CEA, Evry, France) for the three family members II-4, II-7 and III-4 using an Illumina HiSeq2500 platform. DNA was prepared using Illumina TruSeq DNA PCR-Free library preparation kits according to the manufacturer’s instructions. An average sequencing depth of 30x was obtained for each sample.

The VCF files from WES and WGS were analyzed conjointly using a dedicated in-house python pipeline. Variants were filtered according to: 1) their quality (variant quality Phred Qscore >20); 2) the genotype quality (>20) and depth (>5); 3) the predicted consequence in terms of coding substitution and insertion/deletion (frameshift or inframe), splice-site extending to 10 bases, UTR regions, and 1Kb upstream and downstream; 4) their frequency (minor allele frequency <0.003) based on information available in the public genome aggregation database (gnomAD) and 1000 genomes project version as of august 2015; 5) their segregation with the disease in the five family members analyzed; 6) their localization in a gene involved in at least one of the seven pathways in which ADH genes are involved (Reactome (<https://reactome.org>) which are cholesterol biosynthesis (R-HSA-191273), regulation of cholesterol biosynthesis by SREBP (R-HSA-1655829), clathrin-mediated endocytosis (R-HSA-8856828), vesicle-mediated transport (R-HSA-5653656), transport of small molecules (R-HSA-382551), digestion and absorption (R-HSA-8963743), bile acid, and bile salt metabolism (R-HSA-194068)).

Finally, variants in candidate genes were sequenced in the whole family to test their segregation with the disease under the hypothesis of autosomal dominant inheritance.

4.3. Positional Cloning

In parallel with WES and WGS, a positional cloning approach using a genome wide scan in 14 family members was performed with 1035 polymorphic microsatellite markers from deCODE Genetics, Iceland. Parametric linkage analyses were performed with accepted parameters for ADH which were dominant transmission of the trait, penetrance of 0.6 for heterozygotes and a frequency of the disease allele of 0.01%. The power of the family for linkage was evaluated using the FastSlink v2.51 software [44]. We used Pedcheck [45]to detect Mendelian inheritance errors. SuperLink v1.5v [46] and SimWalk v2.91 [47] software were used to compute two-point and multipoint LOD scores. All these software were run using the easyLinkage Plus v5.00 package [48].

4.4. Sequencing and in silico analysis of the variants

The coding exons of *LRP6*, *CYP7A1* and *LDLRAP1* and their flanking exon-intron boundaries (100 kb surrounding each exon boundary) were sequenced by Sanger or by next generation sequencing [49]. The coverage of the 160 unrelated ADH probands was > 99% for the coding bases of *LRP6* and *CYP7A1* gene, and 90% for *LDLRAP1*. The reference sequences were NM\_002336.3 for *LRP6*, [NM\_000780.4](https://www.ncbi.nlm.nih.gov/nuccore/NM_000780.4) for *CYP7A1* and [NM\_015627.3](https://www.ncbi.nlm.nih.gov/nuccore/NM_015627.3) for *LDLRAP1* (GRCh37/hg19 in UCSC Genome Browser).

Novel variants were mapped to known functional domains obtained by the UniProtKB database (UniProt Knowledgebase; [www.uniprot.org](http://www.uniprot.org)). The presence and frequency of these variants in a control group representative of the French population were verified using the French Exome Project database (FREX; [www.france-genomique.org/bases-de-donnees/frex-the-french-exome-project-database/)](http://www.france-genomique.org/bases-de-donnees/frex-the-french-exome-project-database/)). Their frequency in the general population was taken from the Genome Aggregation database (gnomAD; <http://gnomad.broadinstitute.org/>). Their pathogenicity was evaluated using the Varsome tool (The Human Genomic Variant Search Engine; <https://varsome.com>) [50] according to ACMG guidelines [51] and CADD score (Combined Annotation Dependent Depletion; <https://cadd.gs.washington.edu/snv>). In addition, the *in silico* prediction tools PolyPhen-2 (Polymorphism Phenotyping version 2; <https://genetics.bwh.harvard.edu/pph>), Provean (Protein Variation Effect Analyzer; <https://provean.jcvi.org>), ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>), and SpliceAI (<https://spliceailookup.broadinstitute.org>) were used.

4.5. Construction of LRP6 structural models

Structural models of the wild-type and mutant human LRP6-E3E4 (PDB ID 3S8Z) were generated using PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC (<http://www.pymol.org>).

4.6. Weighted polygenic risk score (wPRS) calculation

For each individual, the wPRS was calculated using the weighted sum of the beta coefficient reported by the GLGC of the risk allele for the four selected SNPs plus the two *APOE* SNPs [26,52]. wPRS were then compared to those of 3020 normocholesterolemic men and women of European ancestry from the UK Whitehall II (WHII) cohort study (SE Humphries and M Futema, personal communication). The probability of monogenic FH gradually increases for deciles under V, whereas scores in the top four deciles are associated with a high probability of polygenic hypercholesterolemia.

4.7. Sterol and bile acid measurements

Markers of cholesterol absorption (campesterol, sitosterol, cholestanol), cholesterol synthesis (lathosterol, lanosterol, desmosterol), and cholesterol degradation to bile acids (7α-hydroxy-cholesterol, 27-hydroxy-cholesterol) as well as bile acids were measured in patient’s serum with gas chromatography-mass spectrometry-selected ion monitoring as previously described [53,54]. Values of non-cholesterol sterols and oxysterols were corrected for cholesterol concentration (R\_sterols).

4.8. Site-directed mutagenesis

The coding sequence of *LRP6* (LRP6-WT) (NM\_002336) was cloned into the 4.7 kb vector PCMV6XL4 (PcM) which encodes ampicillin resistance and is suitable for mammalian cell overexpression assays. The PcM and LRP6-WT plasmids were purchased from OriGene® Technologies. Mutated plasmids were generated separately by site-directed mutagenesis using Agilent® Technologies QuickChange II XL Site-Directed Mutagenesis Kit according to manufacturer instructions. Briefly, the mutant strand was synthesized by a thermal cycling reaction using high fidelity DNA polymerase and complementary mutagenic primers. This reaction was followed by *DpnI* digestion of the parental methylated and hemi-methylated DNA. The DNA vector containing the desired variant was then amplified into XL10-Gold® Ultracompetent Cells by ThermoFisher® Scientific and extracted using the NucleoBond® Xtra Midi Plus kit by Machery-Nagel. The constructs were verified by Sanger sequencing with primers spanning the coding sequence of *LRP6*.

4.9. Cell culture and transfection

HuH7 cells were provided by Gael NICOLAS, and Hek293T cells were provided by ThermoFisher Scientific. HuH7 cells and HEK293T cells were authenticated by Eurofins. Cells were cultured in 1X Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS and 1% antibiotic-antimycotic from Gibco® by ThermoFisher® Scientific, at 37°C under 5% CO2 in a humid atmosphere. Routine passage was every 3 days. For all experiments, HuH7 and HEK293T were used at passages two to 10. About 800,000 HEK293T cells were seeded in six-well plates and maintained in complete DMEM medium for 24 hours before transfection. Cells were then co-transfected with the empty vector PcM, LRP6-WT or mutated plasmids, and a plasmid containing cyan fluorescent protein (CFP) or pSF-CMV-FrCFP from Oxford Genetics® to assess transfection rate by fluorescence-activated cell sorting (FACS) analysis. Cell transfections with a total of 3 µg of DNA per well were done with Lipofectamine® LTX and PlusTM Reagent from Invitrogen® by ThermoFisher® Scientific in DMEM medium supplemented with 10% FBS. Twenty-four hours post-transfection, cells were starved for 24 hours in serum-free media.

*LRP6* silencing was achieved using a Silencer® Select siRNA targeting human *LRP6* (siLRP6) from Ambion. A siNeg from Eurogentec® was used as a negative control. The siLRP6 sequence and siNeg sequence were not homologous to any other human gene sequence according to BLAST analysis. HuH7 cells were transfected with siLRP6 or siNeg using the reverse transfection method. Briefly, the siRNA molecule was diluted in 1X OptiMem Medium from Gibco® by ThermoFisher® Scientific in each well of a Biocoat Collagen I Cellware six-well plate and combined with diluted Lipofectamine from Invitrogen® by ThermoFisher® Scientific to form complexes. About 400,000 HuH7 cells were suspended in DMEM medium then added directly to the lipofectamine-siRNA complexes. Transfected HuH7 cells were maintained in the transfection medium until analysis.

Human EBV-transformed B-lymphocytes were cultured in suspension in RPMI-1640-glutamax medium supplemented with 10% FBS and 1% antibiotic-antimycotic from Gibco® by ThermoFisher® Scientific at 37°C under 5% CO2 in a humid atmosphere. The medium was partially renewed every three to seven days. About 2x106 cells were seeded in 24-well plates and starved for 24 hours in serum-free media before FACS analysis or LDL-Bodipy treatment. About 5x106 cells were seeded in 24-well plates. Each cell line was seeded in triplicate and starved for 24 hours in serum-free media before RNA extraction.

4.10. LRP6 and LDL receptor cell surface expression

Human LRP6 APC-conjugated antibody was purchased from R&D Systems, and its specificity was confirmed *in vitro* by ELISA. The isotype control of LRP6, the PE mouse anti-human LDLR and its isotype control were purchased from BD Biosciences®.

Cell surface expression of LRP6 and the LDL receptor in non-permeabilized and transfected HEK293T cells was analyzed by FACS using antibodies that recognize the extracellular region of human LRP6 and human LDLR. Cell surface expression of LDL receptor in human EBV-transformed B-lymphocytes was analyzed by flow cytometry using an antibody that recognized the extracellular region of the human LDL receptor. Forty-eight hours post-transfection with LRP6-WT or mutated plasmid, HEK293T cells were washed with 1X PBS, incubated with both LRP6 and LDL receptor antibodies for 30 minutes at 4ºC then analyzed by FACS using a LSRII from BD Bioscience®. Twenty-four hours after incubation in serum-free medium, human EBV-transformed B-lymphocytes were washed, incubated with LDL receptor antibody for 30 minutes at 4ºC and analyzed on a BD Accuri™ C6 flow cytometer.

Cell viability was assessed by LiveDead® staining from Invitrogen® by ThermoFisher® Scientific. Median fluorescence intensity of 100,000 events was acquired for each sample but only the median fluorescence intensity of living cells is analyzed. The assay was performed independently five times.

4.11. LDL uptake

Low-Density Lipoprotein from Human Plasma and Bodipy ® FL complex (LDL-Bodipy) were purchased from ThermoFisher® Scientific. Uptake of LDL in non-permeabilized and transfected HEK293T and HuH7 cells was analyzed by FACS using LDL-Bodipy. Forty-eight hours post-transfection with LRP6-WT or mutated plasmid and 72 hours post-transfection with siRNA, HEK293T and HuH7 cells were incubated in serum-free medium with 10 µg/mL LDL-Bodipy for 4 hours at 37ºC. After 4 hours of incubation, the medium was removed and the cells were washed twice with ice-cold 1X PBS. Labeled HEK293T and HuH7 cells were analyzed by FACS. Cell viability was assessed by LiveDead® in FACS analysis. Median fluorescence intensity of 50,000 siRNA transfection events in HuH7 and 100,000 plasmid transfection events in HEK293T was acquired for each sample but only the median fluorescence intensity of living cells is analyzed. Each assay was done in triplicate and the triplicate assays were replicated independently three times.

LDL binding and uptake in human EBV-transformed B-lymphocytes was analyzed by FACS using LDL-Bodipy. After incubation of the human EBV-transformed B-lymphocytes in serum-free medium for 24 hours, 10 µg/mL LDL-Bodipy was added and the incubation was prolonged by four hours at 4°C for the binding experiment or at 37ºC for the uptake experiment. The cells were washed and analyzed on a BD Accuri™ C6 FACS. Cell viability was assessed by LiveDead® staining from Invitrogen® by ThermoFisher® Scientific. Median fluorescence intensity of 100,000 events was acquired for each sample but only the median fluorescence intensity of living cells was analyzed. The assay was performed independently five times.

4.12. Protein extraction and western-Blot

Transfected HEK293T cells were lysed in Pierce® RIPA buffer ThermoFisher® Scientific supplemented with HaltTM Protease & Phosphatase Single-Use Inhibitor cocktail (100X) from ThermoFisher® Scientific. Proteins were extracted by centrifugation for 15 minutes at 14,000 g at 4°C. The total protein concentration was quantified on Tecan® infinite 200 Pro using a PierceTM BCA Protein Assay kit from ThermoFisher® Scientific.

Western blot assays were performed following the standard protocol. Equal quantities of protein extracts (2.5 μg) were loaded onto 4-20% Mini-Protean® TGXTM precast protein gels from BioRad®, separated by electrophoresis then transferred to Amersham Hybond™ - P PVDF membrane from GE Healthcare®. The membrane was blocked with 10% non-fat dry milk in TBST 0.1% for one hour at room temperature then incubated overnight at 4°C with primary with recombinant anti-LRP6 antibody (ab134146) from Abcam®. The membrane was washed three times with 0.1% TBST followed by the addition of peroxidase-conjugated affiniPure Goat anti-Rabbit IgG secondary antibody from Jackson ImmunoResearch for one hour at room temperature. The membrane was then washed three times with 0.1% TBST, treated with the ClarityTM Western ECL Substrate from BioRAD® and detected using the iBrightTM FL1500 imaging system from ThermoFisher® scientific. Protein was quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA). As an internal reference, ß-actin was detected using a monoclonal anti-ß-actin-peroxidase antibody (AB3854) from Sigma-Aldrich. The results presented were representative of three independent experiments.

4.13. Total RNA extraction, RT and real-time PCR quantification

Total RNA was extracted from transfected HuH7 cells and human EBV-transformed B-lymphocytes using the RNeasy Mini Kit from Qiagen® according to manufacturer instructions. RNA was eluted into RNase-free water, measured by Nanodrop spectrophotometer by Thermo Scientific® to determine concentration and quality then stored at -80ºC until use. cDNA was produced using random primers and SuperScriptTM II Reverse Transcriptase from Invitrogen® by ThermoFisher® Scientific.

Messenger RNA expression levels of the *LRP6, LDLR, HMGCR, SREBP2,* and *PCSK9* genes were assessed using specific primers in HuH7 cells transfected with siRNA. mRNA expression levels of the *LRP6* gene were assessed in human EBV-transformed B-lymphocytes. Primers of target genes were mixed with Absolute Blue qPCR Mix, SYBR Green, ROX from ThermoFisher® Scientific, and the cDNA solution was diluted to 2 ng/µl. Reactions were run in triplicate for each cDNA on an Applied® Biosystems StepOnePlusTM Real-Time PCR System. The data were analyzed using the StepOne software v2.3 and Microsoft Excel. Threshold cycle (CT) values were used to calculate the relative quantification (RQ) of gene expression using the comparative CT (∆∆CT) method. Data were normalized using *POLR2A* as a housekeeping gene. Observed differences were considered significant when the RQ was below 0.5 or above 2 [55]. The experiments were done in triplicate.

4.14. Statistical analysis

All variables were expressed as mean ± standard deviation and represent the results of four independent experiments. Bonferroni's Multiple Comparison Test in one-way ANOVA was used to assess differences between two groups. To ensure that variances were not significantly different between groups, the Bartlett's test was used. A probability value of *p* < 0.05 was considered significantly different. GraphPad Prism® software was used for the statistical analysis and to generate graphs.

**Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1, Figure S1: Distribution of the probands in the 10 deciles of the weighted Polygenic Risk Score in the UK Whitehall II (WHII) cohort study, Figure S2: Conservation of the mutated amino acid in *LRP6*, Figure S3: Expression of genes implicated in cholesterol metabolism in HuH7 cells transfected with siLRP6, Figure S4: LDL binding and uptake in HEK293T after transfection with empty vector, LRP6-WT or mutated plasmid (cells harvested without trypsin), Figure S5: Effects of transfection with WT or mutated LRP6 on membrane expression of LDL receptor; Table S1: Variants identified in HC438 family by WES and WGS, Table S2: Linkage analysis of the variants identified in HC438 family by WES and WGS.

**Author Contributions:** Funding acquisition: C.B., M.A.F. and M.V. Conception of the work: M.V., M.A.F., and C.B. Acquisition and analysis of data for: *Probands and Family Recruitment:* P.M., M.D-F., S.C., M.F., C.Y., V.C., J.F., J-M.L., and J-P.R.; *Whole Genome and Exome sequencing:* B.F., A.B., R.O., and J-F.D.; *WGS and WES Data analysis:* A.P.; *Positional Cloning:* M.V.; *Sanger and next-generation sequencing:* Y.G, Y.A, Y.A-K., M.V., and M.D-F.; *In silico analysis of the variants:* Y.G.; *Construction of LRP6 structural models:* A.D., G.B., M-S.G., and B.C.; *wPRS calculation:* Y.G, M.V and M.D-F.; *Sterol and bile acid measurements:* D.L.; *Site-directed mutagenesis:* Y.G. and S.E.B.; *Cell culture and transfection:* Y.G., S.E.B., P.E.K, M.A., and G.N.; *Receptor cell surface expression and LDL Uptake:* Y.G., A.L, M.L-B., M.A., and M.V.; *Western-blot and real-time PCR:* Y.G, Y.A., M.A., and Y.A-K.; *Statistical analyses:* Y.G, and M.V. Drafting the work: Y.G., M.V. and M.A.F. Revising the work critically: All.

All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by grants from Leducq Foundation (FLQ # 13CVD03), The Laboratory of Excellence GENMED (Medical Genomics) grant no. ANR-10-LABX-0013 managed by the National Research Agency (ANR) part of the Investment for the Future program, The national project CHOPIN (CHolesterol Personalized Innovation) grant by the National Research Agency (ANR-16-RHUS-0007), INSERM (Institut National de la Santé et de la Recherche Médicale).

Youmna GHALEB is supported by a grant from Lefoulon-Delalande Foundation and The International Atherosclerosis Society (IAS). Yara Azar and Yara Abou-Khalil are supported by grants from Lebanese National Council for Scientific Research (CNRS-L) and Council of Research of Saint-Joseph University of Beirut, Lebanon. Yara Azar is supported by a grant from AMGEN (PCSK9013). Yara ABOU-KHALIL was supported by grants from Ministère de l’Education Nationale et de la Technologie (France) and Nouvelle Société Francophone de l’Athérosclérose (France). Geneviève BERNARD received a Research Scholar Junior 1 award from the Fonds de Recherche du Québec - Santé (FRQS) (2012-2016) and the New Investigator Salary Award from the Canadian Institutes of Health Research (2017-2022).

**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki and received IRB approval by the French Consultative Committee for the Protection of Persons in Biomedical Research, Paris, Necker (research project trial #05-07-06).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study in agreement with French bioethics laws

**Data Availability Statement:** All data have been included in the article.

**Acknowledgments:** We thank all family members and the proband cohort for participating in the study. We thank Pr. Sekar KATHIRESAN, Dr. Gina PELOSO and Dr. Nathan STITZIEL (Broad Institute of MIT, Boston, MA USA) for their help in the exome sequencing experiments. Pr. Sekar KATHIRESAN was supported byNIH R01 HL107816. We acknowledge the use of the bioresources of the Necker DNA Biobank (BB-033-00065). We thank Silvia Friedrichs for technical assistance in sterol and bile acid analyses by GC-MS.

**Conflicts of Interest:** The authors declare no conflict of interest related to the topic of the paper.

References

1. Nordestgaard, B.G.; Chapman, M.J.; Humphries, S.E.; Ginsberg, H.N.; Masana, L.; Descamps, O.S.; Wiklund, O.; Hegele, R.A.; Raal, F.J.; Defesche, J.C.; et al. Familial Hypercholesterolaemia Is Underdiagnosed and Undertreated in the General Population: Guidance for Clinicians to Prevent Coronary Heart Disease: Consensus Statement of the European Atherosclerosis Society. *Eur. Heart J.* **2013**, *34*, 3478–3490a, doi:10.1093/eurheartj/eht273.

2. Béliard, S.; Boccara, F.; Cariou, B.; Carrié, A.; Collet, X.; Farnier, M.; Ferrières, J.; Krempf, M.; Peretti, N.; Rabès, J.-P.; et al. High Burden of Recurrent Cardiovascular Events in Heterozygous Familial Hypercholesterolemia: The French Familial Hypercholesterolemia Registry. *Atherosclerosis* **2018**, *277*, 334–340, doi:10.1016/j.atherosclerosis.2018.08.010.

3. Defesche, J.C.; Gidding, S.S.; Harada-Shiba, M.; Hegele, R.A.; Santos, R.D.; Wierzbicki, A.S. Familial Hypercholesterolaemia. *Nat. Rev. Dis. Primer* **2017**, *3*, 17093, doi:10.1038/nrdp.2017.93.

4. Beheshti, S.O.; Madsen, C.M.; Varbo, A.; Nordestgaard, B.G. Worldwide Prevalence of Familial Hypercholesterolemia: Meta-Analyses of 11 Million Subjects. *J. Am. Coll. Cardiol.* **2020**, *75*, 2553–2566, doi:10.1016/j.jacc.2020.03.057.

5. Berberich, A.J.; Hegele, R.A. The Complex Molecular Genetics of Familial Hypercholesterolaemia. *Nat. Rev. Cardiol.* **2019**, *16*, 9–20, doi:10.1038/s41569-018-0052-6.

6. Brown, M.S.; Goldstein, J.L. A Receptor-Mediated Pathway for Cholesterol Homeostasis. *Science* **1986**, *232*, 34–47.

7. Goldstein, J.L.; Schrott, H.G.; Hazzard, W.R.; Bierman, E.L.; Motulsky, A.G. Hyperlipidemia in Coronary Heart Disease. II. Genetic Analysis of Lipid Levels in 176 Families and Delineation of a New Inherited Disorder, Combined Hyperlipidemia. *J. Clin. Invest.* **1973**, *52*, 1544–1568, doi:10.1172/JCI107332.

8. Innerarity, T.L.; Mahley, R.W.; Weisgraber, K.H.; Bersot, T.P.; Krauss, R.M.; Vega, G.L.; Grundy, S.M.; Friedl, W.; Davignon, J.; McCarthy, B.J. Familial Defective Apolipoprotein B-100: A Mutation of Apolipoprotein B That Causes Hypercholesterolemia. *J. Lipid Res.* **1990**, *31*, 1337–1349.

9. Abifadel, M.; Varret, M.; Rabès, J.-P.; Allard, D.; Ouguerram, K.; Devillers, M.; Cruaud, C.; Benjannet, S.; Wickham, L.; Erlich, D.; et al. Mutations in PCSK9 Cause Autosomal Dominant Hypercholesterolemia. *Nat. Genet.* **2003**, *34*, 154–156, doi:10.1038/ng1161.

10. Marduel, M.; Carrié, A.; Sassolas, A.; Devillers, M.; Carreau, V.; Filippo, M.D.; Erlich, D.; Abifadel, M.; Marques-Pinheiro, A.; Munnich, A.; et al. Molecular Spectrum of Autosomal Dominant Hypercholesterolemia in France. *Hum. Mutat.* **2010**, *31*, E1811, doi:10.1002/humu.21348.

11. Garcia, C.K.; Wilund, K.; Arca, M.; Zuliani, G.; Fellin, R.; Maioli, M.; Calandra, S.; Bertolini, S.; Cossu, F.; Grishin, N.; et al. Autosomal Recessive Hypercholesterolemia Caused by Mutations in a Putative LDL Receptor Adaptor Protein. *Science* **2001**, *292*, 1394–1398, doi:10.1126/science.1060458.

12. Fernández-Higuero, J.A.; Benito-Vicente, A.; Etxebarria, A.; Milicua, J.C.G.; Ostolaza, H.; Arrondo, J.L.R.; Martín, C. Structural Changes Induced by Acidic PH in Human Apolipoprotein B-100. *Sci. Rep.* **2016**, *6*, 36324, doi:10.1038/srep36324.

13. El Khoury, P.; Elbitar, S.; Ghaleb, Y.; Khalil, Y.A.; Varret, M.; Boileau, C.; Abifadel, M. PCSK9 Mutations in Familial Hypercholesterolemia: From a Groundbreaking Discovery to Anti-PCSK9 Therapies. *Curr. Atheroscler. Rep.* **2017**, *19*, 49, doi:10.1007/s11883-017-0684-8.

14. Khalil, Y.A.; Rabès, J.-P.; Boileau, C.; Varret, M. APOE Gene Variants in Primary Dyslipidemia. *Atherosclerosis* **2021**, *328*, 11–22, doi:10.1016/j.atherosclerosis.2021.05.007.

15. Tada, H.; Kawashiri, M.-A.; Nohara, A.; Inazu, A.; Mabuchi, H.; Yamagishi, M. Impact of Clinical Signs and Genetic Diagnosis of Familial Hypercholesterolaemia on the Prevalence of Coronary Artery Disease in Patients with Severe Hypercholesterolaemia. *Eur. Heart J.* **2017**, *38*, 1573–1579, doi:10.1093/eurheartj/ehx004.

16. Reeskamp, L.F.; Volta, A.; Zuurbier, L.; Defesche, J.C.; Hovingh, G.K.; Grefhorst, A. ABCG5 and ABCG8 Genetic Variants in Familial Hypercholesterolemia. *J. Clin. Lipidol.* **2020**, *14*, 207-217.e7, doi:10.1016/j.jacl.2020.01.007.

17. Chora, J.R.; Alves, A.C.; Medeiros, A.M.; Mariano, C.; Lobarinhas, G.; Guerra, A.; Mansilha, H.; Cortez-Pinto, H.; Bourbon, M. Lysosomal Acid Lipase Deficiency: A Hidden Disease among Cohorts of Familial Hypercholesterolemia? *J. Clin. Lipidol.* **2017**, *11*, 477-484.e2, doi:10.1016/j.jacl.2016.11.002.

18. Pullinger, C.R.; Eng, C.; Salen, G.; Shefer, S.; Batta, A.K.; Erickson, S.K.; Verhagen, A.; Rivera, C.R.; Mulvihill, S.J.; Malloy, M.J.; et al. Human Cholesterol 7alpha-Hydroxylase (CYP7A1) Deficiency Has a Hypercholesterolemic Phenotype. *J. Clin. Invest.* **2002**, *110*, 109–117, doi:10.1172/JCI15387.

19. Go, G. Low-Density Lipoprotein Receptor-Related Protein 6 (LRP6) Is a Novel Nutritional Therapeutic Target for Hyperlipidemia, Non-Alcoholic Fatty Liver Disease, and Atherosclerosis. *Nutrients* **2015**, *7*, 4453–4464, doi:10.3390/nu7064453.

20. Go, G.-W.; Mani, A. Low-Density Lipoprotein Receptor (LDLR) Family Orchestrates Cholesterol Homeostasis. *Yale J. Biol. Med.* **2012**, *85*, 19–28.

21. Tomaszewski, M.; Charchar, F.J.; Barnes, T.; Gawron-Kiszka, M.; Sedkowska, A.; Podolecka, E.; Kowalczyk, J.; Rathbone, W.; Kalarus, Z.; Grzeszczak, W.; et al. A Common Variant in Low-Density Lipoprotein Receptor-Related Protein 6 Gene (LRP6) Is Associated with LDL-Cholesterol. *Arterioscler. Thromb. Vasc. Biol.* **2009**, *29*, 1316–1321, doi:10.1161/ATVBAHA.109.185355.

22. Mani, A.; Radhakrishnan, J.; Wang, H.; Mani, A.; Mani, M.-A.; Nelson-Williams, C.; Carew, K.S.; Mane, S.; Najmabadi, H.; Wu, D.; et al. LRP6 Mutation in a Family with Early Coronary Disease and Metabolic Risk Factors. *Science* **2007**, *315*, 1278–1282, doi:10.1126/science.1136370.

23. Montazeri-Najafabady, N.; Dabbaghmanesh, M.H.; Mohammadian Amiri, R. The Association of LRP6 Rs2302685 (V1062I) Polymorphism with the Risk of Hyperlipidemia in Iranian Children and Adolescents. *Ann. Hum. Genet.* **2018**, *82*, 382–388, doi:10.1111/ahg.12254.

24. Lange, L.A.; Hu, Y.; Zhang, H.; Xue, C.; Schmidt, E.M.; Tang, Z.-Z.; Bizon, C.; Lange, E.M.; Smith, J.D.; Turner, E.H.; et al. Whole-Exome Sequencing Identifies Rare and Low-Frequency Coding Variants Associated with LDL Cholesterol. *Am. J. Hum. Genet.* **2014**, *94*, 233–245, doi:10.1016/j.ajhg.2014.01.010.

25. Wang, J.; Dron, J.S.; Ban, M.R.; Robinson, J.F.; McIntyre, A.D.; Alazzam, M.; Zhao, P.J.; Dilliott, A.A.; Cao, H.; Huff, M.W.; et al. Polygenic Versus Monogenic Causes of Hypercholesterolemia Ascertained Clinically. *Arterioscler. Thromb. Vasc. Biol.* **2016**, *36*, 2439–2445, doi:10.1161/ATVBAHA.116.308027.

26. Futema, M.; Shah, S.; Cooper, J.A.; Li, K.; Whittall, R.A.; Sharifi, M.; Goldberg, O.; Drogari, E.; Mollaki, V.; Wiegman, A.; et al. Refinement of Variant Selection for the LDL Cholesterol Genetic Risk Score in the Diagnosis of the Polygenic Form of Clinical Familial Hypercholesterolemia and Replication in Samples from 6 Countries. *Clin. Chem.* **2015**, *61*, 231–238, doi:10.1373/clinchem.2014.231365.

27. Rabès, J.-P.; Béliard, S.; Carrié, A. Familial Hypercholesterolemia: Experience from France. *Curr. Opin. Lipidol.* **2018**, *29*, 65–71, doi:10.1097/MOL.0000000000000496.

28. Varret, M.; Abifadel, M.; Rabès, J.-P.; Boileau, C. Genetic Heterogeneity of Autosomal Dominant Hypercholesterolemia. *Clin. Genet.* **2008**, *73*, 1–13, doi:10.1111/j.1399-0004.2007.00915.x.

29. Sjouke, B.; Defesche, J.C.; Hartgers, M.L.; Wiegman, A.; Roeters van Lennep, J.E.; Kastelein, J.J.; Hovingh, G.K. Double-Heterozygous Autosomal Dominant Hypercholesterolemia: Clinical Characterization of an Underreported Disease. *J. Clin. Lipidol.* **2016**, *10*, 1462–1469, doi:10.1016/j.jacl.2016.09.003.

30. Elbitar, S.; Susan-Resiga, D.; Ghaleb, Y.; El Khoury, P.; Peloso, G.; Stitziel, N.; Rabès, J.-P.; Carreau, V.; Hamelin, J.; Ben-Djoudi-Ouadda, A.; et al. New Sequencing Technologies Help Revealing Unexpected Mutations in Autosomal Dominant Hypercholesterolemia. *Sci. Rep.* **2018**, *8*, 1943, doi:10.1038/s41598-018-20281-9.

31. Tada, H.; Kawashiri, M.-A.; Nomura, A.; Teramoto, R.; Hosomichi, K.; Nohara, A.; Inazu, A.; Mabuchi, H.; Tajima, A.; Yamagishi, M. Oligogenic Familial Hypercholesterolemia, LDL Cholesterol, and Coronary Artery Disease. *J. Clin. Lipidol.* **2018**, *12*, 1436–1444, doi:10.1016/j.jacl.2018.08.006.

32. Haralambos, K.; Whatley, S.D.; Edwards, R.; Gingell, R.; Townsend, D.; Ashfield-Watt, P.; Lansberg, P.; Datta, D.B.N.; McDowell, I.F.W. Clinical Experience of Scoring Criteria for Familial Hypercholesterolaemia (FH) Genetic Testing in Wales. *Atherosclerosis* **2015**, *240*, 190–196, doi:10.1016/j.atherosclerosis.2015.03.003.

33. Danielsson, H.; Einarsson, K.; Johansson, G. Effect of Biliary Drainage on Individual Reactions in the Conversion of Cholesterol to Taurochlic Acid. Bile Acids and Steroids 180. *Eur. J. Biochem.* **1967**, *2*, 44–49.

34. Shefer, S.; Hauser, S.; Bekersky, I.; Mosbach, E.H. Biochemical Site of Regulation of Bile Acid Biosynthesis in the Rat. *J. Lipid Res.* **1970**, *11*, 404–411.

35. Couture, P.; Otvos, J.D.; Cupples, L.A.; Wilson, P.W.; Schaefer, E.J.; Ordovas, J.M. Association of the A-204C Polymorphism in the Cholesterol 7alpha-Hydroxylase Gene with Variations in Plasma Low Density Lipoprotein Cholesterol Levels in the Framingham Offspring Study. *J. Lipid Res.* **1999**, *40*, 1883–1889.

36. Teslovich, T.M.; Musunuru, K.; Smith, A.V.; Edmondson, A.C.; Stylianou, I.M.; Koseki, M.; Pirruccello, J.P.; Ripatti, S.; Chasman, D.I.; Willer, C.J.; et al. Biological, Clinical and Population Relevance of 95 Loci for Blood Lipids. *Nature* **2010**, *466*, 707–713, doi:10.1038/nature09270.

37. Ye, Z.; Go, G.-W.; Singh, R.; Liu, W.; Keramati, A.R.; Mani, A. LRP6 Protein Regulates Low Density Lipoprotein (LDL) Receptor-Mediated LDL Uptake. *J. Biol. Chem.* **2012**, *287*, 1335–1344, doi:10.1074/jbc.M111.295287.

38. Joiner, D.M.; Ke, J.; Zhong, Z.; Xu, H.E.; Williams, B.O. LRP5 and LRP6 in Development and Disease. *Trends Endocrinol. Metab. TEM* **2013**, *24*, 31–39, doi:10.1016/j.tem.2012.10.003.

39. Mineo, C. Lipoprotein Receptor Signalling in Atherosclerosis. *Cardiovasc. Res.* **2020**, *116*, 1254–1274, doi:10.1093/cvr/cvz338.

40. Tomaszewski, M.; Charchar, F.J.; Barnes, T.; Gawron-Kiszka, M.; Sedkowska, A.; Podolecka, E.; Kowalczyk, J.; Rathbone, W.; Kalarus, Z.; Grzeszczak, W.; et al. A Common Variant in Low-Density Lipoprotein Receptor-Related Protein 6 Gene (LRP6) Is Associated with LDL-Cholesterol. *Arterioscler. Thromb. Vasc. Biol.* **2009**, *29*, 1316–1321, doi:10.1161/ATVBAHA.109.185355.

41. Liu, W.; Mani, S.; Davis, N.R.; Sarrafzadegan, N.; Kavathas, P.B.; Mani, A. Mutation in EGFP Domain of LDL Receptor-Related Protein 6 Impairs Cellular LDL Clearance. *Circ. Res.* **2008**, *103*, 1280–1288, doi:10.1161/CIRCRESAHA.108.183863.

42. Siest, G.; Visvikis, S.; Herbeth, B.; Gueguen, R.; Vincent-Viry, M.; Sass, C.; Beaud, B.; Lecomte, E.; Steinmetz, J.; Locuty, J.; et al. Objectives, Design and Recruitment of a Familial and Longitudinal Cohort for Studying Gene-Environment Interactions in the Field of Cardiovascular Risk: The Stanislas Cohort. *Clin. Chem. Lab. Med.* **1998**, *36*, 35–42, doi:10.1515/CCLM.1998.007.

43. Stitziel, N.O.; Peloso, G.M.; Abifadel, M.; Cefalu, A.B.; Fouchier, S.; Motazacker, M.M.; Tada, H.; Larach, D.B.; Awan, Z.; Haller, J.F.; et al. Exome Sequencing in Suspected Monogenic Dyslipidemias. *Circ. Cardiovasc. Genet.* **2015**, *8*, 343–350, doi:10.1161/CIRCGENETICS.114.000776.

44. Schäffer, A.A.; Lemire, M.; Ott, J.; Lathrop, G.M.; Weeks, D.E. Coordinated Conditional Simulation with SLINK and SUP of Many Markers Linked or Associated to a Trait in Large Pedigrees. *Hum. Hered.* **2011**, *71*, 126–134, doi:10.1159/000324177.

45. O’Connell, J.R.; Weeks, D.E. PedCheck: A Program for Identification of Genotype Incompatibilities in Linkage Analysis. *Am. J. Hum. Genet.* **1998**, *63*, 259–266, doi:10.1086/301904.

46. Fishelson, M.; Geiger, D. Exact Genetic Linkage Computations for General Pedigrees. *Bioinforma. Oxf. Engl.* **2002**, *18 Suppl 1*, S189-198.

47. Weeks, D.E. A Likelihood-Based Analysis of Consistent Linkage of a Disease Locus to Two Nonsyntenic Marker Loci: Osteogenesis Imperfecta versus COL1A1 and COL1A2. *Am. J. Hum. Genet.* **1990**, *47*, 592–594.

48. Lindner, T.H.; Hoffmann, K. EasyLINKAGE: A PERL Script for Easy and Automated Two-/Multi-Point Linkage Analyses. *Bioinforma. Oxf. Engl.* **2005**, *21*, 405–407, doi:10.1093/bioinformatics/bti009.

49. Marmontel, O.; Rollat‐Farnier, P.A.; Wozny, A.-S.; Charrière, S.; Vanhoye, X.; Simonet, T.; Chatron, N.; Collin‐Chavagnac, D.; Nony, S.; Dumont, S.; et al. Development of a New Expanded Next-Generation Sequencing Panel for Genetic Diseases Involved in Dyslipidemia. *Clin. Genet.* *n/a*, doi:10.1111/cge.13832.

50. Kopanos, C.; Tsiolkas, V.; Kouris, A.; Chapple, C.E.; Albarca Aguilera, M.; Meyer, R.; Massouras, A. VarSome: The Human Genomic Variant Search Engine. *Bioinforma. Oxf. Engl.* **2019**, *35*, 1978–1980, doi:10.1093/bioinformatics/bty897.

51. Richards, S.; Aziz, N.; Bale, S.; Bick, D.; Das, S.; Gastier-Foster, J.; Grody, W.W.; Hegde, M.; Lyon, E.; Spector, E.; et al. Standards and Guidelines for the Interpretation of Sequence Variants: A Joint Consensus Recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet. Med. Off. J. Am. Coll. Med. Genet.* **2015**, *17*, 405–424, doi:10.1038/gim.2015.30.

52. Ghaleb, Y.; Elbitar, S.; El Khoury, P.; Bruckert, E.; Carreau, V.; Carrié, A.; Moulin, P.; Di-Filippo, M.; Charriere, S.; Iliozer, H.; et al. Usefulness of the Genetic Risk Score to Identify Phenocopies in Families with Familial Hypercholesterolemia? *Eur. J. Hum. Genet. EJHG* **2018**, *26*, 570–578, doi:10.1038/s41431-017-0078-y.

53. Lütjohann, D.; Hahn, C.; Prange, W.; Sudhop, T.; Axelson, M.; Sauerbruch, T.; von Bergmann, K.; Reichel, C. Influence of Rifampin on Serum Markers of Cholesterol and Bile Acid Synthesis in Men. *Int. J. Clin. Pharmacol. Ther.* **2004**, *42*, 307–313, doi:10.5414/cpp42307.

54. Šošić-Jurjević, B.; Lütjohann, D.; Renko, K.; Filipović, B.; Radulović, N.; Ajdžanović, V.; Trifunović, S.; Nestorović, N.; Živanović, J.; Manojlović Stojanoski, M.; et al. The Isoflavones Genistein and Daidzein Increase Hepatic Concentration of Thyroid Hormones and Affect Cholesterol Metabolism in Middle-Aged Male Rats. *J. Steroid Biochem. Mol. Biol.* **2019**, *190*, 1–10, doi:10.1016/j.jsbmb.2019.03.009.

55. Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods San Diego Calif* **2001**, *25*, 402–408, doi:10.1006/meth.2001.1262.